Biochemical Evidence for Cholinergic Activity in Retinal Blood Vessels

Gabryleda Ferrari-Dileo, E. Barry Davis, and Douglas R. Anderson

Blood vessels from human, cat, pig and bovine retinas were analyzed for their contents of choline acetyltransferase (ChAT) and muscarinic binding sites. ChAT was measured by the synthesis of $^3$H-acetylcholine in the presence of $^3$H-acetyl CoA and choline. Muscarinic binding sites were determined by the specific binding of $^3$H-quiniudinyl benzylate ($^3$H-QNB). Tissue levels of ChAT varied from 39-850 nmol/g/hr, the lowest values being in human tissues. Muscarinic binding sites were less different (0.5 to 1.8 fmol/mg net weight) among the species studied, being the highest values in human retinal vessels. It appears that retinal blood vessels not only have sites to bind acetylcholine, perhaps to mediate physiologic responses, but might be capable of supplying acetylcholine for local vascular tone control. Invest Ophthalmol Vis Sci 30:473-477, 1989

Retinal vessels are known to respond to local chemical factors such as CO$_2$ and O$_2$, but after finding a physiological response to exogenous angiotensin$^2$ and the presence of the enzyme responsible for its synthesis,$^3$ we have been interested in the potential role of various hormones and neurotransmitters in retinal and optic nerve vasculatures.

These vessels do not have a cholinergic or adrenergic innervation, at least in cats, monkeys and humans.$^4$ In spite of this, we have found all the four basic types of adrenergic receptors in the retinal vessels, suggesting that potentially these vessels might respond to endogenous and exogenous catecholamines.$^5$ In keeping with our interests, and considering that retinal vessels very much resemble cerebral vessels, we also wanted to investigate the possibility of the presence of cholinergic mechanisms in these vessels. We used $^3$H-quiniudinyl benzylate ($^3$H-QNB) to test the presence of muscarinic receptors. We also looked for choline acetyltransferase (ChAT; EC 2.3.1.6) by using $^3$H-acetyl coenzyme A ($^3$H-AchCoA) and choline to measure the synthesis of acetylcholine in the retinal vessels of several mammals, including humans.

Choline acetyltransferase synthesizes acetylcholine from the precursors acetyl coenzyme A and choline in cholinergic nerves which also contain acetylcholinesterase (AChE) required for the catabolism of acetylcholine. ChAT is also found in non-nerve tissue such as placenta$^8$ and endothelial cells from cerebral blood vessels,$^9$ where its presence reflects the capacity for acetylcholine synthesis in cell types other than cholinergic neurons.

We conducted the same tests in the nonvascular elements of the retina of the same species as a control, since it is well known that the neural elements of mammalian retina possess muscarinic receptors and ChAT activity.$^{10}$

Materials and Methods

All applicable federal guidelines and the ARVO Resolution on the Use of Animals in Research were followed, and approval of the University of Miami Animal Experimentation Committee was obtained. Bovine and swine eyes were obtained at a local abattoir 1 to 2 hr postmortem. Cat eyes were obtained by enucleation from animals under surgical anesthesia. Human eyes were donated from the Florida Lions Eye Bank.

Tissue Isolation

Vessels were separated and isolated from the retinas as already described.$^6$ In brief, retinal vessels are separated from the retinas by hand-driven homogenization, and further purified by filtration and two-step centrifugation. The supernatant retina homogenates obtained after the vessel isolation were also used in the study as control nonvascular retinal tissue. The

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**Table 1. Specific $^3$H-QNB binding (fmol/mg wet membranes)**

<table>
<thead>
<tr>
<th></th>
<th>Bovine</th>
<th>Swine</th>
<th>Cat</th>
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<tbody>
<tr>
<td>Retina</td>
<td>4.6 ± 0.5 (12)</td>
<td>2.5 ± 0.1 (9)</td>
<td>4.6 ± 0.4 (3)</td>
</tr>
<tr>
<td>Vessels</td>
<td>0.5 ± 0.1 (9)</td>
<td>0.9 ± 0.1 (8)</td>
<td>0.8 ± 0.2 (5)</td>
</tr>
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</table>

Values are the mean ± SEM. Numbers in parenthesis are the number of determinations performed in two different experiments.

**Assay for Muscarinic Receptors**

Binding to the vascular and the retinal membranes was performed as described by others. In brief, 5-10 mg of membranes from retinal vessels and retinal homogenates were incubated with 10 mM sodium phosphate with 1 mM EDTA, pH 7.4, with 1 nM ($-$)$^3$H quinuclidinyl benzilate ($^3$H-QNB) (84.9 Ci/mmol, New England Nuclear Corp., Boston, MA) for 45 min at 37°C. An additional set of equal number of tubes for nonspecific binding contained 1 μM unlabelled (±)QNB. Membranes were collected on glass fiber filters, rinsed with buffer at 4°C, dried and counted for radioactivity by liquid scintillation spectroscopy. The Kd for QNB for muscarinic receptors is 5-8 pM in a variety of animal tissues, so 1 nM ($-$)$^3$H-QNB occupies more than 99% of all known muscarinic receptors.

**Assay for Choline Acetyltransferase**

The assay was performed as previously described. In brief, 5-10 mg of membranes from retina and retinal vessels were treated with 0.5% Triton X-100 to release enzyme activity. The tissue fractions were then incubated for 15 min at 37°C in a mixture containing: 0.3 M NaCl, 0.05 M NaH$_2$PO$_4$/Na$_2$HPO$_4$, 0.02 M Na$_3$EDTA, 0.0001 M physostigmine salicilate (Sigma Chemical Co., St. Louis, MO), 0.01 M choline bromide (Sigma Chemical Co.), and 0.056 M $^3$H-acetylCoenzyme A (4 Ci/mmol, New England Nuclear, diluted with unlabelled AcetylCoA), at pH 7.4 in a final volume of 0.25 ml. The reaction was interrupted by rapid dilution with ice-cold 0.01 M phosphate buffer (pH 7.4). The newly synthesized $^3$H-acetylcholine was extracted as a complex with sodium tetraphenylboron formed in the butyronitrile organic phase, and counted by liquid scintillation spectroscopy. As controls, samples from retina and retinal vessels membranes of each one of the species studied were boiled before the addition of the incubation cocktail. The cpm's obtained from these heat-inactivated samples were considered as "background" or "control," and as such were subtracted from total activity values.

**Autoradiography**

Bovine retinas were frozen in liquid nitrogen as soon as removed from the eyes. Eight micron sections were obtained in a cryostat at −20°C and apposed to gelatin-coated slides. Sections were dried at 4°C under reduced pressure and stored at −70°C until used.

Sections were incubated with $^3$H-QNB (with (±)QNB for nonspecific binding) under the same conditions as those described under "Assay for Muscarinic Receptors." After the last ice-cold rinse, sections were dried and apposed to NTB-2 emulsion-covered coverslips and left in the dark at 4°C for 4 to 6 weeks. Autoradiographs were developed in D-19, fixed and stained with hematoxylin for microscopic observation.

Results are expressed as the mean ± standard error of the mean of several determinations performed in two individual and different experiments, unless otherwise stated.

**Results**

**$^3$H-QNB Binding**

The specific binding of the $^3$H-QNB was determined in both the retinal and vascular fractions of each of the species studied. The nonspecific binding was 30-50% of the total binding in the vascular fraction and between 13-26% of the total binding for the neural retina fraction. In Tables 1 and 2 the values of the specific bindings in both fractions for all the species studied are shown. Figure 1A shows a representative autoradiograph of a bovine retinal vessel cross-section incubated with $^3$H-QNB and exposed after 6 weeks. Silver grains are in higher proportion in the retina surrounding the vessels than in the vascular component, in agreement with the finding of $^3$H-QNB in the membranes. In both tissues, however, the density of the silver grains is considerably lower when an excess of (±)QNB is present (Fig. 1B, nonspecific binding) as expected from a displacement of $^3$H-QNB from specific binding sites.
Choline Acetyltransferase Activity

The enzymatic activity was determined in the same fractions as the $^3$H-QNB binding for all the species studied. The amount of $^3$H extracted in heat-inactivated samples from bovine, swine and cat vascular and retinal fractions was considered background and as such subtracted from totals of radioactivity extracted from each fraction in each tissue to obtain the values shown in Table 3. Table 4 shows the values for each fraction of each one of the human donors. Since the yield of tissue for each donor was very low, heat-inactivated samples were not run as with the other three species. To obtain the values shown in Table 4, the mean of the heat-inactivated values from the bovine and swine tissues were used as background.

It appears that human vascular tissue has the highest values for $^3$H-QNB binding among all the species, although lower than those found in the respective human neural retina. On the other hand, human vascular tissue has the lowest values for ChAT activity but higher than in the respective neural retinas. We cannot explain the wide variation in ChAT values; it might correlate with pathological or abnormal neurological states. At the moment, this remains speculative because of the low number of cases and because we did not have access to detailed clinical history of the donors. The finding of higher values in the vascular fraction than in the retinal fraction in humans serves to show that the values obtained for the vascular fraction are not contaminants of retinal elements in the isolated vascular preparation. The purity of the vascular preparation was also shown previously by scanning electron microscopy. Figure 2 shows a comparison of $^3$H-QNB binding and ChAT activity between vascular and retina fractions for all the species studied.

Discussion

Specific $^3$H-QNB binding sites and ChAT activity have been found in the vessels isolated from bovine, pig, cat and human retinas. $^3$H-QNB is a highly specific cholinergic antagonist widely used to characterize muscarine receptors in a great variety of tissues, including vascular tissues. The presence of QNB binding sites in retinal vessels implies that a dilator or constrictor response might occur, providing the binding sites are physiologic receptors and that acetylcholine is present in the sur-

<table>
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<th>Species</th>
<th>Retina</th>
<th>Vessels</th>
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<tbody>
<tr>
<td>Bovine</td>
<td>253±17 (2)*</td>
<td>2555±320 (6)*</td>
</tr>
<tr>
<td></td>
<td>234±10 (2)*</td>
<td>850±320 (6)*</td>
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<td>Pig</td>
<td>201±2 (2)*</td>
<td>1601±38 (12)*</td>
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<tr>
<td></td>
<td>189±8 (2)*</td>
<td>421±33 (12)*</td>
</tr>
<tr>
<td>Cat</td>
<td>200±3 (2)*</td>
<td>433±113 (6)*</td>
</tr>
<tr>
<td></td>
<td>167±20 (2)*</td>
<td>519±100 (7)*</td>
</tr>
</tbody>
</table>

* Values in heat-inactivated samples ("background").
† Values in samples after subtraction of "background."
Numbers in parentheses are the number of determinations performed in two different experiments.
roundings in quantities enough to bind to these sites. However, how can acetylcholine be present around these vascular binding sites if the retinal vessels seem to lack any type of autonomic innervation? It has been shown that acetylcholine released from the heart has a half-life of less than 5 seconds. During strong vagal stimulation, enough acetylcholine from the heart could reach the eye in that time. However, it is important to remember that retinal vessels have tight junctions and lack fenestrations, so circulating acetylcholine could reach smooth muscle cells only in the presence of a retina–blood barrier breakage, or reach muscarinic receptors if located in endothelial cells, as shown for other arterioles.

One possibility is that these muscarinic binding sites have no physiological role at all in the retinal vascular bed. Perhaps they are simply present in all vessels and participate in normal physiologic reactions only in those anatomic sites where the agonist is present. Another possibility is a functional input from cholinergic cells in the inner layer of the retina. The vertebrate retinas contain large amounts of acetylcholinesterase (the enzyme responsible for acetylcholine degradation). This makes us think that acetylcholine produced in the retina might not reach the vessels in its intact form. However, the lack of evidence for retino-vascular interactions makes it difficult to assess any possible participation of retinal–acetylcholine in controlling retinal circulation. An alternative possibility is that there is a source of agonist besides neural and circulating hormones, namely local synthesis of acetylcholine in the vascular tissue itself, independent from any neural input. Tissues without an established cholinergic innervation have been shown to be able to synthesize and store acetylcholine.

In particular, endothelial cells from the cerebral vascular bed, closely related to retinal vessels, have been shown to have the enzyme and the capacity to synthesize acetylcholine.

In this study we show that retinal vessels are indeed able to synthesize acetylcholine when acetyl coenzyme A and choline are provided. This new findings suggest that vessels from bovine, cat, pig and human retinas have the enzymatic machinery, choline acetyltransferase (ChAT), to provide acetylcholine for muscarinic binding sites in their cells. We were not able to find any cholinergic innervation in bovine or human retinal vessels by using the acetylcholinesterase staining method (unpublished observations), which indicates that the ChAT activity found in the vascular fraction is located in muscle or endothelial cells themselves. Few investigators have previously reported finding ChAT activity in non-neural tissue, and our data suggest that acetylcholine may have more widespread physiologic presence than previously suspected. We have not been able to show the exact localization for ChAT yet, but we hope to localize it by immunocytochemical binding of the enzyme in future studies.

The physiological or pathological implications of the synthesis of acetylcholine by retinal vessels are not easy to assess. The muscarinic receptors might be located in the endothelial layers and contribute to local control of vascular tone, as has been shown for peripheral arteries. Similarly, the local production of acetylcholine might be a normal physiological response to maintain an equilibrium with vasoconstrictor actions, or it might occur only when damage to the endothelium requires a local protection from hypoxia. However, all these explanations will remain speculations until a physiological response is recorded, and there is documentation of the conditions that may activate or inactivate the synthesizing enzyme.

The presence of muscarinic receptors and ChAT activity in the neural retina (nonvascular fraction) were expected findings since acetylcholine is a neurotransmitter in amacrine cells.

**Key words:** retinal blood vessels, muscarinic receptors, choline acetyltransferase, QNB-binding sites, autoradiography

### Table 4. ChAT activity in human tissues (nmol/g/hr)

<table>
<thead>
<tr>
<th>Retina</th>
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<tbody>
<tr>
<td>1</td>
<td>14.0</td>
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<tr>
<td>2</td>
<td>115.0</td>
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<td>3</td>
<td>56.0</td>
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<td>4</td>
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Fig. 2. Relative values of \(^3\)H-QNB binding and ChAT activity in retinal vessels. Values were taken from Tables 1–4 and processed as: specific value vessel/specific value retina \(\times 100\), for each species.
Acknowledgments
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References